C2 Domain Function in Healthy and Diseased Brain

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Chapter 1
General Introduction

Regulated secretion

A variety of physiological processes are dependent on regulated secretion of signaling molecules, for example pancreatic beta cells releasing insulin into circulation, cytokine signaling by immune cells in response to injury or infection, and of course neuronal communication. Neurotransmitter release is certainly the most intricately regulated system and is still far from completely understood. One of the most fundamental mechanisms in regulated secretion is orchestrated by proteins that translate the calcium signal into membrane fusion.

C2 domain proteins

C2 domains are conserved protein structures initially described in the PKC (protein kinase C) family, and named conserved domain 2[1]. They were later identified in over 200 mammalian proteins.

Synaptotagmins are possibly the most well-known and numerous of the C2 domain proteins. Seventeen mammalian synaptotagmin proteins have been identified so far[2]. Synaptotagmins share the following molecular organization: an N-terminal transmembrane region is connected through a variable length linker to tandem C2 domains (Fig. 1A). Synaptotagmin-1 (Syt-1) has been studied extensively, and many of its functions and corresponding amino acid residues have been characterized. It is a calcium sensor for synchronous neurotransmitter release in the synapse, through calcium-dependent interactions with anionic lipids and SNARE complex proteins[3,4]. Mice lacking Syt-1 die shortly after birth; their synaptic vesicle release in response to an action potential occurs in an asynchronous manner[5].

Several members of the synaptotagmin family have similar functions. Syt-2 has a complementary expression profile to Syt-1 and also mediates synchronous neurotransmitter release[6]. Syt-7 regulates calcium-dependent vesicle release from endocrine cells[7]. Syt-9 is responsible for fast calcium-triggered exocytosis, and is capable of rescuing the synchronous phase of release in Syt-1 deficient cells[8]. Syt-10 is implicated in calcium-independent spontaneous release[9]. In general, it appears that most synaptotagmins mediate membrane fusion, with different calcium affinities and fusion kinetics.

Doc2B is a sensor for spontaneous vesicle release[10]. It is activated by lower calcium concentrations than Synaptotagmin-1 (apparent calcium affinity of ~175nM for Doc2B versus ~10µM for Syt-1). A homologous protein, Doc2A, can fulfill the same functions as Doc2B *in vitro*[10] and in living cells[11,12]. Doc2 proteins share many functional properties with Synaptotagmins, such as calcium-dependent phospholipid and SNARE protein binding, and positive regulation of synaptic vesicle fusion. In place of the transmembrane domain of Syt-1, Doc2 proteins have an N-terminal MID domain (Fig. 1B) that mediates binding to presynaptic proteins Munc-13[13].

Piccolo is a component of the presynaptic active zone, the site where synaptic vesicles become mature before being released (Fig. 2). Piccolo is fundamental for the formation and organization of the active zone, and it is positioned in a way to control the access of synaptic vesicles to the presynaptic plasma membrane[14]. Piccolo is a multi-domain protein of high molecular weight (over 500kDa), and has two C2 domains at its C-terminal. The C2A domain of Piccolo binds phospholipid membranes[15], while the C2B domain properties are largely unknown.

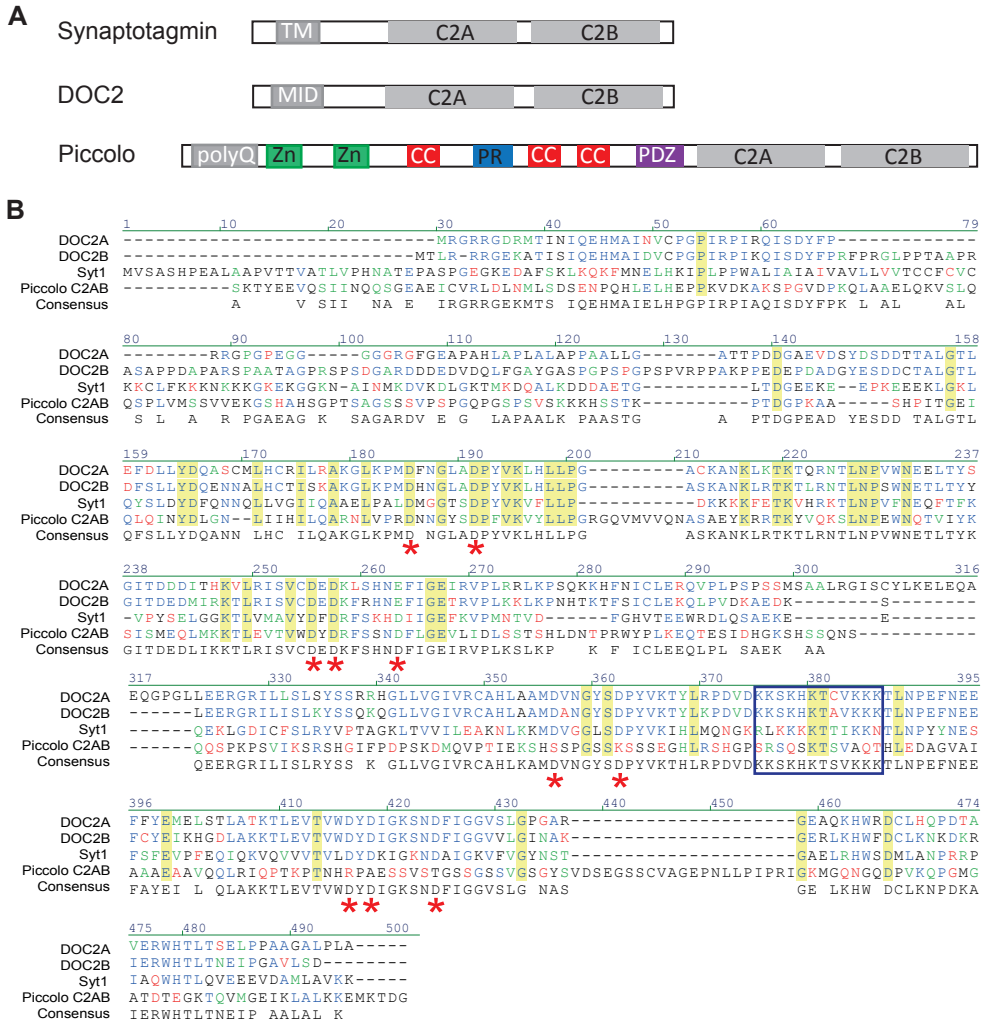

Figure 1. A. Domain structure of Synaptotagmin, DOC2 proteins, and Piccolo; TM – transmembrane, MID – Munc13-binding, Zn - Zinc Finger, CC - coiled coil, PR - Proline Rich domains. **B.** Alignment of full length Doc2A, Doc2B, Synaptotagmin-1 and C2AB domains of Piccolo. Aminoacid residues conserved in all 4 proteins have yellow background, black - non similar residues, blue – conservative, green – block of similar, red – weakly similar. Blue box denotes the polybasic stretch; red asterisks – aspartic acid residues that bind calcium ions.

Typically, C2 domains are known to bind calcium ions and phospholipid membranes; however several C2 domains are known that bind neither calcium nor lipids. It is possible that their functions are mediated by protein-protein interactions. The number of C2 domains in different proteins varies between one and seven. In proteins containing one C2 domain this usually has the function of localizing the protein to the membrane, often in response to calcium elevation (e.g. PKC, PLC). Many proteins contain more than one C2 domain; presence of multiple C2 domains is usually associated with direct role in membrane dynamics and vesicle release. For example, PKC and PLC become localized to the plasma membrane in response to intracellular calcium elevation via their single C2 domain; the enzymatic domains then act on membrane-associated molecules. Proteins belonging to synaptotagmin and ferlin families have between two and seven C2 domains; most of them are known to promote membrane fusion.

Table 1 describes proteins with two or more C2 domains and their main properties. Note that intrinsic calcium affinity is in general 10-100 times higher in presence (in almost all cases 25% phosphatidylserine and 75% phosphatidylcholine), than in absence of phospholipids. The latter is referred to as intrinsic Ca^{2+} affinity. Also note that the C2A domains of different synaptotagmins generally have higher calcium affinities than the C2B domains.

Structurally, a C2 domain consists of eight β-strands arranged in a barrel-like structure with a total length of approximately 125–130 amino acids[45,46]. C2 domains feature a Ca^{2+}-binding pocket in which calcium ions are bound by five negatively charged amino acids (aspartates) (Fig 1B). Ca^{2+} binding reverses their net negative charge, enabling an interaction of surrounding hydrophobic loops with phospholipid membranes[47,48]. Upon calcium binding, Syt-1 and Doc2B promote assembly of the SNARE complex[10].

Even if C2 domains are mostly known for their calcium and/or membrane binding properties, their protein binding partners have been less studied. Syt-1 and Doc2A/B proteins bind SNARE complex proteins via the so-called polybasic stretch[49], with several conserved Lysine residues (Figure 1B). The neuronal SNARE complex consists of three membrane-associated proteins: Vamp-2/Synaptobrevin-2 on the synaptic vesicle, Syntaxin-1 and SNAP-25 associated with the plasma membrane (Fig 1). Complete assembly of the alpha-helices of these three proteins is essential for synaptic vesicle fusion with the plasma membrane[50]. Several C2 domains (e.g. Syt-1, Munc-13) are able to form homo- or heterodimers[51,52]. This might lead either to cooperative reinforcement of function or auto-inhibition[53]. Little is known so far about other protein binding partners of C2 domains.

Synaptotagmin-1 and Doc2 proteins mediate coupling of the Ca^{2+} signal to vesicle secretion. Many different mechanisms have been proposed to explain how C2 domains regulate membrane fusion. These can be subdivided in two main mechanisms: via the SNARE proteins, or through membrane remodeling. Synaptotagmin/Doc2 may act by (a) disengaging from the SNAREs, and relieving the block (fusion clamp model), (b) binding to the SNAREs, thus displacing the inhibitory complexin and/or promoting zippering, (c) membrane binding in close proximity to partially assembled SNARE complex, destabilizing the bilayer at the fusion site, (d) increasing curvature stress by displacing lipids in the monolayer of the plasma membrane facing the vesicle, and (e) cross-linking the plasma membrane and the synaptic vesicle, and providing a positive electrostatic potential to compensate the negative charge of the phospholipids.

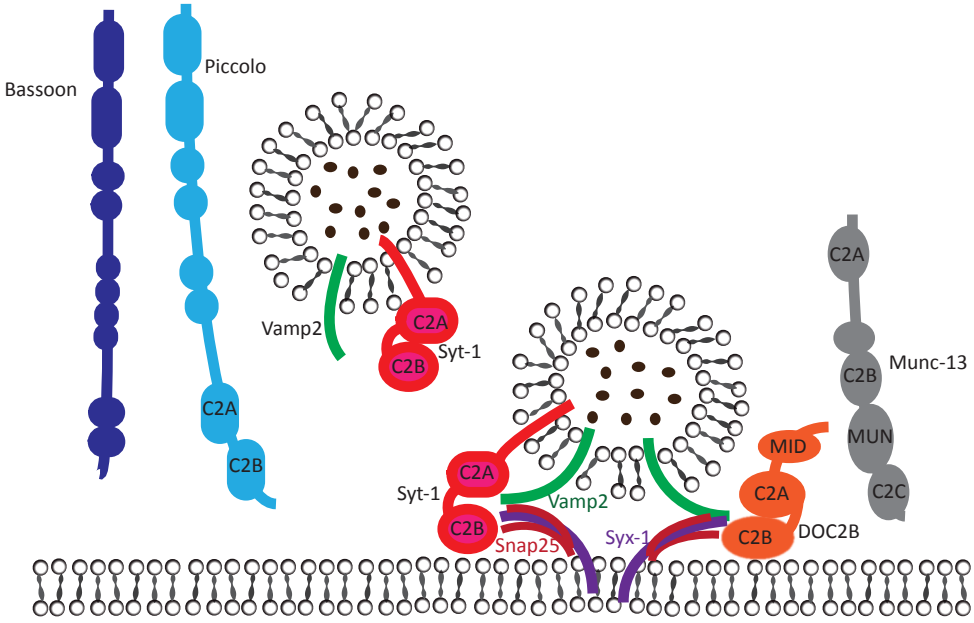

Figure 2. Presynaptic compartment with selected proteins. Synaptic vesicles fusion with the plasma membrane is mediated by the SNARE complex proteins Vamp-2, syntaxin-1 and SNAP25. Syt-1 and DOC2B are the calcium sensors that activate synaptic vesicle fusion. Piccolo is closely related to Bassoon, but contains additional C2 domains. Munc-13 functions by priming synaptic vesicles for release, and has three C2 domains.

Table 1. C2 doman proteins

Protein	Apparent calcium affinity	PIP_2 binding	SNARE binding	Tissue distribution, subcellular localization	Function	Notes	References
Syt-1	~10 μM	Yes	Yes	SVs, Endocrine cells	Ca^{2+}-sensor for fast exocytos.; neurite outgrowth, axonal repair	N-terminal N-glycosylation	5,16,17
Syt-2	~20 μM	Yes	Yes	SVs, LDCVs; Mast cell lysosomes	Ca^{2+}-sensor for fast exocytos.; lysosomal exocytosis	N-terminal N-glycosylation	6,18,19
Syt-3	~2 μM	Yes	No	Plasma membrane	Ca^{2+}-sensor for exocytosis	Disulfide bonds at N-terminus	18,20,21
Syt-4	No calcium binding	No	No	Postsynaptic	Not essential for survival	Asp to Ser substitution in C2A domain loops	22
Syt-5	~3 μM	Yes	Unknown	Plasma membrane	Ca^{2+}-sensor for exocytosis?	Disulfide bonds at N-terminus	17,18,20
Syt-6	~2 μM	Unknown	Unknown	Plasma membrane	Ca^{2+}-sensor for exocytosis?	Disulfide bonds at N-terminus	17,20
Syt-7	~1.5 μM	Yes	Yes	LDCVs; neuronal plasma membrane	Ca^{2+}-sensor for LDCV exocytosis	Multiple splice isoforms	7,18,20,23,24
Syt-8	No calcium binding	No	No	Kidney, GI tract, sperm acrosomes	Fusogenic?		17,20
Syt-9	~6 μM	Unknown	No	SVs	Ca^{2+}-sensor for fast exocytosis		8,25
Syt-10	~8 μM	Yes	Unknown	Olfactory bulb neurons, plasma membrane	Ca^{2+}-sensor for fast exocytosis	Disulfide bonds at N-terminus	9,17,18
Syt-11	No calcium binding	Unknown	Predicted	Hippocampus: SVs, plasma membrane, postsynapse; endocrine cells	Similar to Syt-4, but more abundant	Associated with Parkinson's and schizophrenia	20,26
Syt-12	No calcium binding	Unknown	No	SVs	Miniature release?	Phosphorylated by PKA	20,27

Protein	Apparent calcium affinity	PIP_2 binding	SNARE binding	Tissue distribution, subcellular localization	Function	Notes	References
Doc2A	450 nM	Yes	Yes	Neurons and endocrine cells, mast cells	Spontaneous SV release; DCV release	N-terminal Munc13/ Munc18 binding domain	11,12
Doc2B	175 nM	Yes	Yes	Neurons, adipocytes, endocrine cells	Spontaneous SV release; DCV release	N-terminal Munc13/ Munc18 binding domain	10,11,28,29
Doc2C	No calcium binding	Unknown	Unknown	Heart muscle cells	Unknown		30
Rabphilin	Yes	Yes	Yes	Neurons and endocrine cells	SV re-priming	N-terminal Rab3 binding domain	31-33
Munc13	~5 µM	Yes	Yes	Ubiquitous	SV priming	2 or 3 C2 domains	34-36
Piccolo	~4 µM (short); ~147 µM (long)	Yes	No	Neuronal active zone	SV transit from reserve to release-ready pool	Associated with depression; two C2A splice variants	15,37,38
Otoferlin	~2-10 µM	Yes	Yes	Inner Hair cells	SV priming and fusion	6 C2 domains; essential for hearing	39-41
Dysferlin	~1 µm	Yes	Predicted	Myocytes	Membrane repair; trafficking	7 C2 domains; associated with Muscular Dystrophy	42,43

Table based on[44]. Only proteins with tandem C2 domains (at least 2 consecutive C2 domains) were included. Note: proteins whose properties are unknown were excluded from the table: Syt13-17, Esyt1/2/3. Abbreviations: SVs – synaptic vesicles, LDCVs - large dense core vesicles; PKA – protein kinase A; GI – gastro-intestinal.

Induction of membrane curvature by Doc2B and Synaptotagmin-1 may result from the penetration of hydrophobic residues into one leaflet of the membrane to approximately the depth of lipid glycerol backbones[54,55]. This property is shared with other protein domains involved in membrane dynamics, such as BAR and ENTH domains. It has been shown that the insertion of amphipathic helices into monolayers to the level of the glycerol backbones of lipids (the region of maximum rigidity) leads to induction of membrane curvature[56,57]. This is due to the fact that inserted helix expands the area occupied by the lipid polar heads but not the acyl chains, resulting in a void below the helix that must be filled by the acyl chains bending. This bending is propagated through neighboring lipids and induces local curvature of the monolayer on the surface where protein insertion took place. Due to monolayer coupling, the other phospholipid surface follows, so that the entire bilayer is bent. It is possible that C2 domains work in a similar fashion, inserting into the monolayer to a depth that induces maximal local curvature. It is important that not just one, but at least 2 C2 domains are present. Indeed, single calcium-and phospholipid binding C2 domains (e.g. of PKC, or separate C2A or C2B domain of Syt-1) cannot induce membrane curvature[58]. In some proteins, more than two C2 domains are present in tandem. Ferlin family proteins, containing 6 or 7 tandem C2 domains, contribute to synaptic vesicle fusion in specialized cells[39,42].

C2 domains and disease

Many proteins containing C2 domains have essential properties for cell function or survival. Variations in several C2 domain proteins have been proven directly causative or associated with human diseases.

Frameshift or deleterious mutations in dysferlin gene were found to cause limb-girdle muscular dystrophy[59], and Miyoshi myopathy[60]. Dysferlin is a large transmembrane protein composed of a C-terminal transmembrane domain, two DysF domains, and seven C2 domains that mediate lipid- and protein-binding interactions. It is involved in membrane repair in muscle cells through lysosome fusion (that requires calcium- and lipid binding by the C2A domain of dysferlin)[61].

Otoferlin mutations cause autosomal recessive deafness[62]. Otoferlin contains a transmembrane domain and six C2 domains (a splice variant of otoferlin with three C2 domains exists in humans)[63]. All six C2 domains of otoferlin bind calcium, phospholipid membranes and SNARE proteins[41]. Otoferlin is the calcium sensor for vesicle fusion in the cochlear inner hair cells[39], with a mechanism similar to Synaptotagmin-1. Its absence from inner hair cells blocks calcium-dependent exocytosis, and thus transmission of the sound signal.

Frameshift mutations in CC2D2A gene that cause C2 domain deletion were described in autosomal-recessive mental retardation[64] and Meckel syndrome, lethal malformation disorder characterized by encephalocele, polycystic kidney, and polydactyly[65]. Missense mutations in CC2D2A were found in Joubert syndrome, a less severe version of Meckel syndrome[66]. CC2D2A contains one C2 domain, and is believed to function in ciliogenesis and maintenance of ciliary membrane composition[67]. In neurons cilia regulate signaling pathways (sonic hedgehog, wnt) involved in neural stem cell proliferation, cell fate determination and migration, thus playing an important role in brain development[68].

A single nucleotide variation in the C2A domain of Piccolo gene has been identified as risk factor for Major Depressive Disorder in a genome-wide association study[37,69]. This common variation produces a change in amino acid sequence (serine to alanine). This amino acid is located on the surface area of the Piccolo C2 domain, and might play a role in molecular interactions. A high-resolution study strongly supported the idea that this SNP, and not nearby variations in linkage disequilibrium, affects the risk for major depression[69]. Ultimate evidence for a causal involvement, however, requires a reverse genetics approach to study the effect of the polymorphism on PCLO gene function.

Phospholipid membrane components and their interaction with C2 domains

Membrane interaction plays an important role in C2-domain protein function. Most C2 domain proteins are known to bind to anionic phospholipids, such as phosphatidylserine and phosphoinositides. Membrane shape and fusion depends on (local) lipid composition of membranes[70], which in turn determines C2 domain localization and activity[71,72]. Figure 3 shows the chemical structures of the most important phospholipids. Phosphatidylcholine (PC), which is a zwitterionic phospholipid (with zero net chargeis present in high quantities in mammalian plasma membrane (65% of all phospholipids)[73], and is inert to Syt-1 and Doc2B C2 domain binding (certain C2 domains bind specifically to PC, such as phospholipase A C2 domain[74]).

Phosphatidylserine (PtdSer or PS) is the second most abundant phospholid (24% of total cell phospholids). It is important for the function of C2-domain proteins because upon elevation of intracellular calcium C2 domains acquire high affinity for PS[75]. It has a net negative charge of -1. Phosphatidylserine is restricted to the inner leaflet of the plasma membrane (it is kept there with the help of an enzyme called flippase). When a cell undergoes apoptosis, the asymmetry is lost and phosphatidylserine becomes exposed on the surface of the cell[76]. Additionally, PS is enriched on the outer membrane surface of secretory vesicles.

Phosphatidylethanolammine (PE) is also highly abundant in mammalian cells, with high levels in the brain (up to 45%). It is neutral or zwitterionic phospholipid believed to be involved, among others, in membrane fusion and curvature[77].

Phosphatidylinositol (4,5)-bisphosphate (PIP_2 or PI(4,5)P2) was shown to participate in many cellular processes: regulation of exocytosis[78], endocytosis[78], actin cytoskeleton[79], channel and transporter regulation[80], and it is required for neuronal dense core vesicle exocytosis[81]. The role of PIP_2 in regulating exocytosis can be partially explained by its interaction with syntaxin-1[82]. Like PS, PIP_2 is enriched on the inner leaflet of the plasma membrane. The levels of PIP_2 are under strict and fast regulation, depending upon other things on neuronal activity. Importantly, synaptic vesicle membranes do not contain any PIP_2. Synaptotagmin-1 and Doc2B do not require calcium to bind to PIP_2[83,84]. Their C2 domains bind PIP_2 via the same polybasic stretch that also mediates SNARE complex binding[85-87]. Association of synaptotagmin-1 and Doc2B with PIP_2 is important for fusion of synaptic vesicles, but the exact mode of action is debated. A special feature of PIP_2 is its large headgroup (see also Figure 3) with a high negative charge (between -3 and -5). PIP_2 can act by producing second messengers or by anchoring proteins to the membrane. PIP_2 is hydrolyzed by phospholipase C (PLC), generating the signaling molecules Ins(1,4,5)P3 and diacylglycerol (DAG). It can also be phosphorylated by the class I phosphoinositide 3-kinases to phosphatidylinositol (3,4,5)-triphosphate, which activates various signalling pathways, most notably Akt (protein kinase B)[88].

Methods to study phospholipids in vitro

In the experiments described in this thesis we make extensive use of phospholipid vesicles and supported lipid bilayers. Phospholipid vesicles of known composition are a good model system for the study of membrane properties and protein-membrane interactions. Dehydrated lipids exposed to water will spontaneously form vesicles. These vesicles are typically multilamellar and their size ranges between tens of nanometers to several micrometres. The size and layers of the vesicles depend on the rate of rehydration: the slower, the smaller and thinner the vesicles. Methods such as sonication or extrusion through a porous membrane are needed to break these initial vesicles into smaller, single-walled vesicles of uniform diameter, known as small unilamellar vesicles (SUVs). SUVs are typically between 50 and 200 nm diameter (for comparison, average diameter of a synaptic vesicle is 45 nm).

In the work described here we formed supported lipid bilayers on the surface of polystyrene beads. A supported bilayer is a planar phospholipid structure resting on a solid support. Because of this, only the upper surface of the bilayer is exposed to free solution. This layout has advantages and drawbacks related to the study of lipid

bilayers. One of the greatest advantages of the supported bilayers is their stability (for up to several weeks), even when subject to high flow rates or vibration and eventual holes will not destroy the entire bilayer. On the other hand, membrane dynamics and deformation cannot be completely reproduced using supported bilayers.

Measurement of forces at the molecular scale

Membrane binding is a core feature of C2 domain function. Measurement of the forces of membrane interaction by C2 domains is a powerful tool to achieve better understanding of the role of C2 domains in membrane fusion. Many intracellular processes are driven by molecular forces: cell motility, replication and segregation of DNA, organelle transport and membrane fusion. The scale of forces involved in cellular dynamics is minuscule, correlated with the dimensions of an average protein: several nanometers (1 nm = 10^{-9} m=10 Å). The scale of interactions between molecules is also in the range of nanometers. Weak noncovalent bonds break in the piconewton (1 pN=10^{-12} N) range[89]. Indeed, typical forces for protein-protein interactions range from few piconewton to several hundred piconewton (e.g. streptavidin-biotin interaction, considered a strong intermolecular interaction, is estimated to be around 140-270 pN[90-92].

The main methods to measure forces at molecular level are atomic force microscopy (AFM) and optical tweezers. AFM can measure forces from 10 pN up to 10 nN, whereas this range is between 0.1 and 500 pN for optical tweezers. The temporal resolution of optical tweezers is 10^{-4} sec, and 10^{-3} sec for AFM[93].

Optical tweezers allow no-contact manipulation of small (20nm-5µm) objects. An optical trap is created by focusing a laser with a high numerical aperture objective. Dielectric (polarizable) particles located close to the focus will be pulled towards the centre of the focus, and will experience two forces: scattering (directed along the laser beam propagation) and gradient (directed along the focus gradient) force. These two forces need to be balanced to form a stable trap. For displacements under 100nm from the equilibrium position, the force is linearly proportional to displacement, and the optical trap is then approximated to a spring. The stiffness of the trap, or spring constant, depends on how tightly the laser is focused, the laser power and the polarizability of the trapped object. If a spherical bead is trapped in equilibrium in a laser beam, a position detector (normally a photodiode) will register a symmetric interference pattern, equivalent to zero force. Displacement of the bead from the equilibrium position will result in an asymmetric interference profile, and a detector signal proportional to bead displacement. This method of force measurement is called back focal plane interferometry[94]. To accurately deter-

mine displacement and force, it is necessary to calibrate the position detector and measure the stiffness of the optical trap. The position detector is calibrated by moving the trapped bead through a known distance while recording the position signal. One of the most common methods to calibrate the stiffness of the optical trap is to measure the power spectrum of the thermal fluctuations of the trapped bead.

Certain double C2 domain proteins, like Synaptotagmin-1 and Doc2B are called fusogenic because their activity is essential for forms of membrane fusion in living systems and sufficient in cell-free systems[84]. In a purified system, without the presence of the SNARE proteins, Syt-1 and Doc2B are able to induce membrane curvature[95,96], and cross-link free liposomes[58,97]. A long-standing question is whether these proteins are actively inducing force on membranes, and are contributing to the energy-intensive phospholipid rearrangements that lead to fusion.

Aims and outline of the thesis

The general goal of this work was to increase the level of understanding of how C2 domains contribute to neurotransmission and overall brain function. For that purpose two different approaches were used. First, we studied naturally occurring C2 domains mutations, identified in genetic studies of brain disorders (major depression and cognitive disability). This not only allowed improving our understanding of C2 domain function, but also the function of the proteins in which they occur (Piccolo and CC2D2A, respectively). We also used a biophysical approach to the study of normal C2 domain function: we developed and applied a new method to measure C2 domain interaction with membranes. This approach gave us insight into the fundamental working mechanism of Doc2B, its C2 domains, and their role in membrane fusion. These two approaches can be combined in the future to further deepen our knowledge of C2 domains and their role in health and disease.

In **Chapter 2** we investigated the functional effects of a genetic variation in the C2A domain of Piccolo, previously associated with major depressive disorder in human subjects[37]. We created a knock-in mouse model that carried the variation found in patients. While we found small but significant changes in certain cellular parameters, the behavioral parameters of these mice were unchanged.

In **Chapter 3** we describe the development of a new method to study the interaction of C2 domains with phospholipid membranes. We used optical tweezers based method to perform real-time force spectroscopic measurements and fluorescence microscopy imaging. Double C2AB domains of Doc2B were able to induce hemifusion of membranes in a calcium- and phosphatidyserine dependent manner, without the SNARE proteins.

Chapter 4 is dedicated to the protein CC2D2A, implicated in at least three severe neurological disorders in humans. In all cases the mutations resulted in deletion of the C2 domain of CC2D2A. The function of the protein is little understood, while nothing is known about the properties of its C2 domain. We have studied cellular expression of deletion mutants of CC2D2A, as well as calcium-dependent phospholipid binding by its C2 domain.

Chapter 5 contains a detailed description of the methods for production and purification of recombinant C2 domains, which were indispensable for all the projects described in this thesis, particularly for the highly sensitive force measurements.

Finally, in **Chapter 6** we provide further interpretation to the results presented in the experimental chapters, as well as suggestions for future research.

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